COMMONWEALTH OF AUSTRALIA

Patents Act 1952

CONVENTION APPLICATION FOR A STANDARD PATENT

I/WE, SANOFI, a French societe anonyme of 40, avenue George V, 75008 Paris, France

hereby apply for the grant of a Standard Patent for an invention entitled:
STABILIZED PROTOPLASTS

which is described in the accompanying complete specification.

This application is made under the provision of Part XVI of the Patents Act 1952 and is based on an application for a patent or similar protection made

in France on 24 December 1987
No. (87 18186)

~~in~~ ~~on~~
~~No.~~ (

~~My~~/Our address for service is:
F.B. RICE & CO.,
28A Montague St.
Balmain NSW 2041

Dated this 22nd day of December 1988

SANOFI

By: _____
Registered Patent Attorney

To: The Commissioner of Patents
COMMONWEALTH OF AUSTRALIA

AUSTRALIAN — PATENT DECLARATION FORM
(CONVENTION OR NON-CONVENTION)

(Note: This is a comprehensive form, and parts inappropriate to a particular case must be deleted. This declaration must be signed by the applicant(s), if individuals. If applicant is a company, this declaration must be signed by a person on its behalf and the Company seal or stamp should not be applied. No legalisation is required)

Forms 7 and 8

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INSTRUCTIONS

DECLARATION IN SUPPORT OF A CONVENTION ~~OR NON-CONVENTION~~ APPLICATION FOR A PATENT ~~OR PATENT OF ADDITION~~

(a) Insert No. if available — In support of the application No. (a) ....

(b) Insert full name(s) of applicant(s) — made by (b) SANOFI, 40, avenue George V, 75008 Paris, France

(c) Insert title of invention — for a patent/ ~~patent of addition~~ for an invention entitled (c) STABILIZED PROTOPLASTS

PLEASE ADD NAME AND TITLE OF PERSON SIGNING, LEGIBLY.

(d) Insert full name(s) of declarant(s) who must be PERSON or PERSONS, NOT a corporate body (See head note) — I, (d) Michel de Haas of and care of the applicant company

(e) Insert address(es) of declarant(s) — of (e) ....

do solemnly and sincerely declare as follows:—

(f) Delete entirely if applicant is corporate body. — ~~1. (f) I am/We are the applicant(s) for the patent/patent of addition.~~

(g) Delete entirely if applicant is person or persons — 1. (g) I am authorized by the abovementioned applicant for the patent/~~patent of addition~~ to make this declaration on its behalf.

(h) Delete entirely if Convention priority NOT claimed

(i) Insert country in which first basic application was filed.

(j) Insert date of first basic application.

(k) Insert full name(s) of basic applicant(s).

2. The basic application~~(s)~~ as defined by Section 141 of the Act was/~~were~~ made in the following country ~~or countries~~ on the following date~~(s)~~ by the following applicant~~(s)~~ namely:—

(h) in (i) France on (j) 24 December 19 87
by (k) SANOFI
~~in~~ (i) .... ~~on (j)~~ .... 19 ....
~~by~~ (k) ....
~~in (i)~~ .... ~~on (j)~~ .... 19 ....
~~by (k)~~ ....

(l) Delete entirely if applicant(s) NOT inventor(s)

~~3. (l) I am/We are the actual inventor(s) of the invention.~~

(m) Insert full name(s) of actual inventor(s) if applicant(s) NOT inventor(s)

(n) Insert address(es) of actual inventor(s) if applicant(s) NOT inventor(s)

3. (m) Michel BROUSSE, Patrick JARA, Christian DESHAYES, Gilles LAGARDE,

of (n) Val de la Saune, STE FOY D'AIGREFEUILLE 31570 LANTA, FRANCE; 27, rue des Iris Jaunes, 31320 CASTANET TOLOSAN, FRANCE; 17, rue des Couteliers 31000 TOULOUSE, FRANCE; 9 Cours Debille 75011 PARIS, FRANCE.

~~is~~/are the actual inventor(s) of the invention and the facts upon which the applicant(s) is/are entitled to make the application are as follows:—

(o) Recite manner in which applicant(s) derive(s) title from actual inventor(s) and/or from basic applicant(s)

(o) the applicant is a person who would if a patent were granted upon an application made by the actual inventors, be entitled to have the patent assigned to it.

(p) Delete entirely if Convention priority NOT claimed

(q) Signature(s) of declarant(s)

(N.B. No seal or stamp impression to be applied)

4. (p) The basic application(s) referred to in paragraph 2 of this Declaration was/were the first application(s) made in a Convention country in respect of the invention the subject of the application.

Declared at Paris this 16th day of December 1988

Michel de HAAS

(12) PATENT ABSTRACT (11) Document No. AU-A-27434/88
(19) AUSTRALIAN PATENT OFFICE

(54) Title
STABILIZED PROTOPLASTS

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(57) Claim

Thus, one feature of the invention relates to compositions of stabilized protoplasts, characterized in that they consist of a suspension of bacterial protoplasts, in the frozen or lyophilized state, in a cryoprotective medium.

The cryoprotective medium used according to the invention is a medium making it possible to conserve the integrity of the protoplasts during freezing, lyophilization and subsequent storage.

This medium is advantageously skimmed milk or an aqueous solution containing a sugar. The sugars appropriate for the purposes of the invention are sugars whose molecules do not penetrate into protoplasts on account of their molecular weight being too large, for example. Sugars particularly valued are the osides, in particular the holosides or polysaccharides, such as sucrose, maltose, cellobiose, melibiose, lactose, trehalose or the maltodextrins.

The amount of sugar in the cryoprotective medium is chosen so as to have an osmotic pressure of this latter close to that of the intracellular content of the protoplasts, which in turn depends on the bacteria from which the protoplasts are formed. For example, for protoplasts derived from Streptococcus thermophilus aqueous solutions containing osides, having a molecular weight of about 340 and whose actual osmolality of oside is measured by a cryoscopic osmometer is about 650 milliosmoles, are advantageously used.

1. Composition of stabilized protoplasts, characterized in that it consists of a suspension of bacterial protoplasts, in the frozen or lyophilized state, in a cryoprotective medium.

8. Process for the preparation of a composition according to any one of claims 1 to 7, characterized in that it consists in the following steps :

a) protoplast formation from bacteria

b) freezing of the suspension of protoplasts obtained in step a) in a cryoprotective medium.

c) optionally, lyophilization of the frozen suspension thus obtained.

11. Process for the conservation of bacterial protoplasts, characterized in that it comprises the following steps :

a) introduction of the protoplasts into a cryoprotective medium,

b) freezing of the resulting suspension,

c) storage of the frozen suspension until it is used.

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COMPLETE SPECIFICATION

(ORIGINAL)

Application Number	:	
Lodged	:	
Complete Specification Lodged	:	
Accepted	:	
Published	:	
Priority	:	24 December 1987
Related Art	:	

Name of Applicant	:	SANOFI
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Actual Inventor/s	:	Michel BROUSSE Patrick JARA Christian DESHAYES
Address for Service	:	F.B. RICE & CO. Patent Attorneys 28A Montague Street, Balmain N.S.W. 2041

Complete Specification for the invention entitled:

STABILIZED PROTOPLASTS

The following statement is a full description of this invention including the best method of performing it known to us/me:-

The present invention relates to compositions of stabilized bacterial protoplasts, a process for the preparation of these compositions and a process for the preservation of the bacterial protoplasts.

In EP-A-O 246 163, a process for the treatment of milk for cheese making was described. This process consists in inoculating the milk with bacterial protoplasts before the addition of rennet.

The bacterial protoplastsemployed in this process are prepared according to known methods of the prior art, which consist essentially in removing the bacterial cell wall. According to one of these methods, lysozyme is used for the preparation of suspensions of protoplasts.

These suspensions of protoplasts are to be used as soon as possible after the addition of lysozyme to the bacterial suspension.

For a differed use, such suspensions must be stabilized, for example, by incorporating into the protoplast suspension an osmotic stabilizer, such as sucrose or lactose, at a concentration of O.5M, or by reconstituting, with the aid of the said protoplast suspension and skimmed milk powder, a milk, the osmotic pressure of which is close to that of the intracellular content of the protoplasts.

However, such stabilized suspensions can only be stored for a limited period.

In an unexpected manner, compositions of bacterial protoplasts have now been discovered within which the protoplasts are stabilized, i.e. during prolonged periods of storage they do not undergo degradation which, as a result of involvement of their cell membrane, could lead to lysis.

Thus, one feature of the invention relates to compositions of stabilized protoplasts, characterized in that they consist of a suspension of bacterial protoplasts, in the frozen or lyophilized state, in a cryoprotective medium.

The cryoprotective medium used according to the invention is a medium making it possible to conserve the integrity of the protoplasts during freezing, lyophilization and subsequent storage.

This medium is advantageously skimmed milk or an aqueous solution containing a sugar. The sugars appropriate for the purposes of the invention are sugars whose molecules do not penetrate into protoplasts on account of their molecular weight being too large, for example. Sugars particularly valued are the osides, in particular the holosides or polysaccharides, such as sucrose, maltose, cellobiose, melibiose, lactose, trehalose or the maltodextrins.

The amount of sugar in the cryoprotective medium is chosen so as to have an osmotic pressure of this latter close to that of the intracellular content of the protoplasts, which in turn depends on the bacteria from which the protoplasts are formed. For example, for protoplasts derived from Streptococcus thermophilus aqueous solutions containing osides, having a molecular weight of about 340 and whose actual osmolality of oside is measured by a cryoscopic osmometer is about 650 milliosmoles, are advantageously used.

Such compositions can be prepared from known bacterial strains. Among these latter, the strains used for fermentation purposes in the agri-foodstuff industries constitute a material of choice. Among these latter, thermophilic strains and mesophilic strains are particularly valued. Strains of lactic bacteria are particularly well suited.

These lactic strains include, in particular, the genera Lactobacillus, Leuconostoc and Streptococcus ; mention may also be made of the alkalizing bacteria of the coryneform group, among which the genus Arthrobacter and the species Brevibacterium linens play a decisive role, as well as the micrococci (genus Micrococcus) or the propionic bacteria (genus Propionibacterium), the action of which is well known for cheeses with internal holes, such as the gruyère of comté.

A second feature of the invention relates to a process for the preparation of compositions of stabilized protoplasts which comprises the following steps :

1) formation of protoplasts from bacteria ;

2) freezing of a suspension of the protoplasts obtained in step 1) in a cryoprotective medium ;

3) if required, lyophilization of the frozen suspension thus obtained.

The formation of protoplasts or protoplastisation is carried out, according to the techniques of the prior art, by the action of enzymes which are capable of breaking down the bacterial cell wall. It is advantageous to work in the presence of lysozyme according to the procedure described in the patent application EP-A-0 246 163 cited in the present description as a reference.

According to a preferred procedure, the formation of protoplasts is carried out in an osmoprotective medium, i.e. a medium making it possible to preserve the integrity of the protoplasts formed. It is thought, in fact, that the osmotic pressure of the latter plays an important role in the stabilization of the protoplasts. Aqueous solutions of sugars are advantageously used, preferably the same solutions as those used as cryoprotective media. The molar concentration of sugar of such solutions is advantageously 0.5M. The suspension of protoplasts thus obtained, stabilized by the osmoprotective medium if required, is then frozen in a cryoprotective liquid such as that defined earlier. Preferably, the cryoprotective medium of step 2) comprises the osmoprotective medium of step 1).

Freezing must necessarily be carried out rapidly so as to avoid the formation of large crystals capable of adversely affecting the cell membrane of the protoplasts.

A preferred freezing treatment consists in placing in a container, the temperature of which is lower than -80°C, cryoprotective medium containing 10^{10} to 10^{12} protoplasts per ml distributed in one or more recipients so as to give rise to a liquid layer of from 0.5 to 2cm deep. Under these conditions, the frozen composition thus prepared acquires a completely crystalline structure within about one hour. This composition can then be lyophilized if required.

The lyophilization must be conducted according to the rules known to the person skilled in the art and must take into account the fragile nature of the biological material under consideration.

Preferably, the frozen suspension is then lyophilized for at least 24 hours in a lyophilizer maintained at -45°C; when lyophilization is complete, the resulting composition is advantageously brought to 20°C in order to remove residual moisture.

The compositions of bacterial protoplasts thus obtained can be utilized in the process according to the patent EP-A-0 246 163.

When frozen compositions are used, it is first advisable to place the recipients containing them at a temperature which allows them to thaw. When lyophilized compositions are used, they should be rehydrated with distilled water.

Another feature of the invention relates to a process for the preservation of bacterial protoplasts characterized in that a) the protoplasts are introduced into a cryoprotective liquid, b) the resulting suspension is frozen and c) the frozen composition is stored up to its use. Storage at -80°C is particularly appropriate.

The preservation process can be improved by subjecting the frozen composition to lyophilization. The lyophilized composition can be stored advantageously at +4°C.

The examples given below illustrate the invention in a non-limiting manner.

EXAMPLE 1

I - PREPARATION OF PROTOPLAST-BASED LYOPHILIZED COMPOSITIONS

1) Strains

8 strains were used. All these strains are known and available to the public ; they may be obtained in particular from the Centre National de Recherches Zootechniques (France) (CNRZ).

Brevibacterium linens (CNRZ 221) (mesophile)

Leuconostoc cremoris (CNRZ 361) (mesophile)

Micrococcus sp. (CNRZ 468)(mesophile)

Propionibacterium freundereichii subsp. shermanii (CNRZ 82) hereafter Propionibacterium(mesophile)

Streptococcus cremoris (CNRZ 106), hereafter S. cremoris (mesophile)

Streptococcus lactis (strain isolated from the lyophilized mixture marketed by the ROGER laboratories under

the designation "Mesophilic bacteria, soft pastes"), hereafter S. lactis (mesophile).

Streptococcus lactis diacetylactis (CNRZ 124), hereafter S. lactis diacetylactis (mesophile).

Streptococcus thermophilus (CNRZ 302), hereafter S. thermophilus (thermophile)

2) Media, solutions and reagents used for the growth of the strains, the formation of the protoplasts as well as freezing and lyophilization.

- Growth media for the strains :

The above-mentioned strains were grown on one of the media given below :

MEDIUM A

(Medium known under the designation M17 described by B. Terzaghi et al. in Applied Microbiology, 23,6, 1975 : 807-813)

Composition :

* Part a :

Papain peptone of soya	(Biokar, reference 116002).....	5 g
Casein meat peptone	(Biokar, reference 104008).....	5 g
Yeast extract	(Difco, reference 0127-05-3)....	2.5 g
Beef extract	(Difco, reference 0126-01-08)...	5 g
Ascorbic acid	.....	0.5 g
Disodium glycerophosphate	.....	1 g
1M $MgSO_4$, $7H_2O$	.....	1 ml
Distilled water to give	.....	950 ml

After adjustment of the pH to 7.2 with 4N NaOH, part a is sterilized by heat treatment (120°C, 20 min).

* Part b:

Lactose	.....	10 g
Distilled water to give	.....	50 ml

The part b is sterilized by filtration through a membrane of porosity 0.22 µm.

The medium A is obtained by adding part b to part a.

MEDIUM B

Bacto tryptic soy broth (Difco, reference 0370-01)..... 40 g

MOPS buffer 3-(N-morpholino) propane-sulfonic acid (Sigma, reference M 1254)..... 2 g

Yeast extract (Difco, reference 0127-05-3)..... 5 g

Distilled water to give.....1000 ml

After adjustment of the pH to 7.2 with 4N sodium hydroxide, the medium is sterilized by thermal treatment (120°C, 20 min).

MEDIUM C

Composition

D-glucose..... 10 g

Beef extract (Difco, reference 0126-01-08) ..... 10 g

Trypsin casein peptone (Biokar, reference 104001)..... 10 g

Yeast extract (Difco, reference 0127-05-3)..... 5 g

Ammonium acetate..... 5 g

Sodium acetate..... 2 g

Polyoxyethylenesorbitan monolaurate (Tween^{R} 80)..... 1 g

$MgSO_4$, $7H_2O$..... 0.2g

$MnSO_4$, $4H_2O$.....0.05g

Trace elements in solution..... 1ml

H_3BO_3..... 30 mg

$MnCl_2$,$4H_2O$..... 70 mg

$ZnCl_2$.....200 mg

Na_2MoO_4,$2H_2O$..... 20 mg

$FeCl_3$,$6H_2O$..... 50 mg

$CuSO_4$,$5H_2O$.....200 mg

Distilled water to give....500 ml

Distilled water to give ..... 1000 ml

The pH is adjusted to 6.7 before sterilization.

The medium is sterilized by thermal treatment (120°C, 20 min).

MEDIUM D

Composition :

- Solution 1

D-glucose..... 10 g

$Na_2HPO_4, 2H_2O$..... 2 g

KH_2PO_4..... 2 g

$MgSO_4, 7H_2O$..... 0.5 g

$MnSO_4, 4H_2O$..... 0.05 g

Distilled water to give .....900 ml

The pH is adjusted to 6.8 before sterilization.

The solution is sterilized by filtration through a membrane of porosity 0.22 µm.

- Solution 2

Composition :

Yeast extract (Difco, reference 0127-05-3).... 10 g

Distilled water to give..... 100 ml

The pH is adjusted to 6.8 before sterilization.

The solution is sterilized by thermal treatment (120°C, 20 min).

The complete medium D is obtained by adding solution 2 to solution 1.

- Solution of egg white lysozyme

Composition :

Lysozyme hydrochloride from the G. ROGER laboratories (enzymatic activity 22,500 SHUGAR units/mg)..... 2.5 g

Distilled water to give..... 100 ml

This solution is sterilized by filtration through a membrane of porosity 0.22 µm.

- Buffer for the preparation of protoplasts

Composition :

Sucrose..... 171 g

NaCl..... 0.584 g

$MgCl_2$..... 1.02 g

100mM tri (hydroxymethyl) aminomethane-HCl buffer,

pH 8 to give..... 1000 ml

The buffer is sterilized by thermal treatment (120°C, 20 min).

- Cryoprotective medium

Skimmed milk is reconstituted by the addition of 930 ml of distilled water to 100 g of Regilait^{R} powder. It is sterilized by thermal treatment (110°C, 20 min).

3) Procedure

* General Principle :

The strains are placed in culture so as to have available a working suspension of each of them.

These working suspensions serve as the starting material for the production of protoplasts. The suspensions of protoplasts obtained are frozen and then lyophilized.

a) Culture of the strains

The conditions of culture are presented in Table 1 below.

TABLE 1

STRAIN	MEDIUM	ATMOS-PHERE	TEMPERATURE (°C)	TIME OF PRE-CULTURE (h)	TIME OF CULTURE (h)
Brevibacterium linens	B	aerobiosis	30	5	12
Leuconostoc cremoris	C	aerobiosis	30	5	18
Micrococcus sp.	B	aerobiosis	30	5	8
Propionibacterium	D	anaerobiosis	30	-	26
S. cremoris	A	aerobiosis	30	5	10
S. lactis	A	aerobiosis	30	5	8
S. lactis diacetylactis	A	aerobiosis	30	5	10
S. thermophilus	A	aerobiosis	37	5	16

For each strain, with the exception of that of Propionibacterium, 10 ml of culture medium are inoculated with about 500 µl of bacterial suspension contained in an ampule frozen at -80°C. After incubation, this preculture is transferred to 500 ml of the same medium. The suspension obtained is incubated under gentle agitation.

The Propionibacterium strain is also cultured starting from a frozen ampule. It is not submitted to a preculture. The medium directly inoculated is incubated without agitation.

Working suspensions (WS) containing about 10^9 bacteria counted as colony forming units (CFU) are harvested. At this stage the culture is at the end of the exponential phase of the growth.

b) Preparation of the protoplasts

1 l of suspension (WS) obtained in a) is centrifuged (4825 g, 30 minutes).

The supernatant (S1) is discarded. The bacterial cake (C_1) is taken up in 300 ml of physiological saline. The suspension (SC1) obtained is centrifuged (4825g, 30 minutes). The supernatant (S2) is discarded. The bacterial cake (C2) is taken up in 300 ml of physiological saline. The suspension (SC2) obtained is centrifuged (4825 g, 30 minutes). The supernatant (S3) is discarded. The bacterial cake (C3) is taken up in 130ml of buffer used for protoplast formation. The suspension (SC3) obtained is then homogenized by stirring.

To 130 ml of the suspension (SC3) which contains about 10^{12} CFU are added 20 ml of the lysozyme solution. The mixture obtained is incubated at 44°C with gentle stirring for 4 hours in the case of the Micrococcus sp. strain and 2 hours for each of the other strains. The suspension obtained (SP1) contains the protoplasts and bacteria with an intact cell wall.

The efficiency of the operations leading to the production of the protoplasts was checked by counting the protoplasts contained in the suspensions (SP1) with the aid of a MALASSEZ cell counter and after dilution in a 0.5M sucrose solution and counting of the residual bacteria after dilution in a 1% solution of SDS (sodium dodecylsulfate). In addition, the number of residual bacteria was estimated after streaking the suspension on an agar-

agar medium. In this way, it has been observed that only one bacterium in 10,000 is resistant to the treatment described above in the case of the WS suspension of S. lactis, only one bacterium in 1,000 in the case of the WS suspensions of Previbacterium linens and only one bacterium in 100 for the WS suspensions of Micrococcus sp. and Propionibacterium.

c) Freezing and lyophilization

The suspension (SP1) obtained in b is centrifuged (at 2830 g for 30 minutes, at +4°C). The supernatant (S4) is discarded. The cake (C4) is taken up in 150 ml of the 0.5M sucrose solution. The suspension obtained (SC4) is centrifuged (at 2830 g, for 30 minutes, at +4°C). The supernatant (S5) is discarded. The (C5) is taken up in 12 ml of skimmed milk.

The suspension obtained (SC5) is homogenized by careful aspiration into, and expiration from a 25 ml pipette, then it is distributed in flasks of the penicillin type such that the suspension has a depth of 5 mm; suitable stoppers are attached to the flasks. The flasks are placed on the trays of a lyophilizer.

The trays are placed in a deep freezer adjusted to -80°C. After being left at this temperature for at least one hour, the trays are introduced into the lyophilizer, the chamber of which has been precooled to -45°C.

The lyophilization is accomplished within 24 hours, at the end of which the product is brought to 20°C. The flasks are closed in the sublimation chamber by lowering the trays.

II - PROPERTIES OF THE LYOPHILIZED COMPOSITIONS

The 8 compositions prepared in I prove to contain a high number of protoplasts per unit volume after rehydration by the addition of physiological saline in a volume equivalent to that in which the composition was contained before lyophilization.

The protoplasts were counted with the aid of a MALASSEZ cell counter and the results below were obtained, the yields recorded expressing the percentage of protoplasts which were resistant to lyophilization.

TABLE 2

	YIELD AFTER LYOPHILIZATION (%)
Brevibacterium linens	41
Leuconostoc cremoris	24
Micrococcus sp.	50
Propionibacterium	33
S. cremoris	53
S. lactis	50
S. lactis diacetylactis	24
S. thermophilus	26

III - UTILIZATION OF LYOPHILIZED COMPOSITIONS BASED ON LYOPHILIZED PROTOPLASTS IN THE MANUFACTURE OF CHEESE

Two assays were carried out, the first relating to the manufacture of a stabilized soft cheese with a mixed rind (1st assay), and the other relating to the manufacture of a soft cheese with a washed rind (2nd assay). These assays were performed 48 hours after the preparation of the stabilized protoplasts.

The manufacturing processes were carried out in the standard manner. The protoplasts were added to the milk in the manufacturing vat before the addition of rennet.

In order to evaluate the action of the protoplasts, the degree of ripening was determined by the measurement of a physical parameter (1st assay). Organoleptic tests (2nd assay) were also performed.

1) Manufacture of a stabilized soft cheese with a mixed rind

a) Manufacturing protocol

The strain of Streptococcus lactis was used. Two suspensions of protoplasts in physiological saline, one containing 10^{12} protoplasts and the other 10^{10} protoplasts, are prepared from a lyophilized composition such as that obtained in paragraph I.2.c.

The assay was set up in two series, differing with respect to each other in the number of protoplasts added per 100 liters of manufacturing milk to which rennet had not yet been added :

* series 1 : 10^{10} protoplasts
* series 2 : 10^{12} protoplasts

Description of the cheese

. Skimmed milk cheese : 25% fat/dry extract

. Form : circular units with a mean weight of 1.5 kg.

Manufacturing parameters

Milk matured at pH 6.4 before addition of rennet.

Inoculation with lactic flora by means of FLORA DANICA® bacteria

Addition of rennet

Removal of small amounts of lactose from the vat

Spontaneous drainage until the pH reaches 5.20

Brine salting to give a salt content of between 1.2 and 1.3%.

Ripening parameters

* Nature of the microbial inoculates :

Inoculation of the milk with micrococci and red ferments

Inoculation on the surface with a microbial cocktail constituted of micrococci, red ferments and spores of Geotrichum candidum

* Ripening process :

Maintenance of the cheeses at 13-14°C for 17 days

Packaging

Maintenance of the cheeses at 8°C.

A control manufacture series, to which protoplasts were not added, was run in parallel with series 1 and 2.

Samples of the cheeses of series 1 and 2 and of those in the control series were taken :

- 48 hours after removal from the brine,
- just before packaging,
- 8 days after packaging,

and 25 days after packaging.

b) Measurements made

The change in the degree of ripening was monitored for 38 days by measuring the freezing point of aqueous extracts prepared from the samples and by calculating their standard deviations from that of distilled water (in the sense of a lowering).

1) Method

The method used is a modification of that described by M. COURROYE (Revue Laitière Française, 462, 1987 : 53-54).

Each sample is treated as described below :

30 to 40 g of material taken by vertical core sampling half-way between the outside edge and the center of each piece of cheese are ground with the aid of a mixer of the food processor type. 10g of the crushed material are suspended in 40 ml of ultra-purified water. This suspension is incubated at 80°C for 20 minutes. The aqueous extract is separated. The insoluble paste formed is washed twice with 5ml of ultra-purified water. Finally, the aqueous extract and the washing waters are pooled and their volume is made up to 50 ml with ultra-purified water.

The total extract thus prepared is filtered through WHATMAN® No. 42 paper. The filtrate is recovered ; it is used for the determination of the degree of ripening. This determination is made indirectly by measuring the freezing point of the filtrate, then by calculating the lowering of the latter with respect to that of distilled water. The higher the degree of ripening, the greater is the lowering.

The apparatus used is an automatic cryoscopic osmometer (Roebling^{R}).

This apparatus measures the freezing point of a 100 µl of filtrate, determines the resulting lowering by comparing it with that of distilled water and, by conversion of the value obtained, indicates directly the osmolality of the filtrate.

The values of osmolality obtained were converted by comparing them with a standard curve constructed from the following data (table 3) in order to be able to compare them with the data of M. COURROYE cited above who expresses the degree of ripening in terms of cryoscopic lowering measured in °C ;

TABLE 3

NaCl SOLUTION CONTAINING X g of NaCl PER kg of WATER	ACTUAL OSMOLALITY millimoles	CRYOSCOPIC LOWERING (°C)
X = 3.087	100	0.186
X = 6.260	200	0.371
X = 9.463	300	0.556
X = 12.684	400	0.741
X = 15.916	500	0.925
X = 19.147	600	1.109
X = 22.380	700	1.292

2) Results

The curves shown in figure 1 present the measurements made for series 1. The curve Y1 corresponds to the test manufacture, and the curve TY1 corresponds to a control manufacture carried out under identical conditions but without addition of protoplasts. It has been verified by a statistical test that the deviations observed between the two values for each pair of measurements, test and control, carried out on the same day are significant.

It is observed that ripening in the test manufacture is always at a more advanced stage than that of the control manufacture.

An identical result was obtained in series 2.

The values measured for this series 2 on the 38th day of ripening are presented in table 4 below :

TABLE 4

	CRYOSCOPIC LOWERING MEASURED ON DAY 38	
	(^{o}C)	(^{o}C)
SERIES No. 1	0.256	0.292
SERIES No. 2	0.247	0.275

It can be seen from this that it is also possible to control the degree of ripening by introducing protoplasts, the ripening being more accelerated, the greater the number of protoplasts added.

2) Manufacture of a soft cheese with a washed rind

a) Manufacturing protocol

Four series of experiments were carried out. In each series, a suspension of protoplasts prepared from a particular strain served to inoculate 100 liters of manufacturing milk to which rennet had not yet been added (table 5).

TABLE 5

STRAIN	NUMBER OF PROTOPLASTS PER 100 LITRES OF MILK
SERIES 1 Streptococcus lactis	10^{13}
SERIES 2 Brevibacterium linens	10^{12}
SERIES 3 Micrococcus sp.	10^{12}
SERIES 4 Streptococcus thermophilus	10^{12}

Description of the cheese

Soft cheese with washed rind,

45% of fat/dry extract,

Form: ciruclar unit with a mean weight of 500 g

Manufacturing parameters

Milk prematured for 14 hours at 11°C with 0.1% of ferments until the pH reached 6.6.

Maturation in the vat for 1h30 at 25°C with 0.1% of ferments

Addition of rennet at pH 6.45,

Cutting of coagulum followed by molding,

Drainage in the mold for 24 h,

Brine treatment for 1 hour (salt concentration : 1.8% in the cheese).

Ripening parameters

Working out of the cheeses

Ripening of the cheeses at 13°C with two washings per week with a salt solution of Brevibacterium linens

Samples were taken after :

- 30 days of ripening,

and 45 days of ripening.

b) Measurements made

Organoleptic tests were carried out on the 30th and 45th day of ripening ;

1. Methods

The tests performed by a panel of four persons consisted of :

* a visual evaluation of the degree of ripening
* a gustatory evaluation of the degree of ripening
* overall evaluation of the appearance of the cheeses.

The results of these tests are presented in tables 6 and 7.

TABLE 6

Organoleptic tests on the thirtieth day of ripening.			
Manufacture	Overall evaluation of appearance of the cheeses	Visual evaluation of the degree of ripening	Gustatory evaluation of the degree of ripening
Control	Normal	Extensive hard core	Aroma not developed
Series 1	Normal	Core slightly hard, much less extensive than that of the control	Aroma and taste developed and characteristic
Series 2	Normal		
Series 3	Normal		
Series 4	Normal		

TABLE 7

Organoleptic tests on the forty fifth day of ripening			
Manufacture	Overall evaluation of appearance of the cheeses	Visual evaluation of the degree of ripening	Gustatory evaluation of the degree of ripening
Control	Marked flowing beneath the rind	Hard core	Flat aroma
Series 1	Normal	Flexible paste Degree of ripening approximately identical with that observed on day 30	Aroma and taste well developed
Series 2	Normal		Tendency to bitterness
Series 3	Normal		Aroma and taste well developed
Series 4	Normal		Aroma and taste well developed

Prior to an analysis of the results, it should be noted that, in the case of a traditional manufacture identical with that employed for the control, the production of a well-developed aroma and taste usually requires ripening for two to two and a half months and that most often certain defects such as a flowing beneath the rind are in fact observed when these cheeses are examined on the 45th day of ripening.

The results presented show that the addition of protoplasts makes it possible to obtain within 30 days cheeses in a state of maturation which can only be obtained after 45 days without protoplasts. The acceleration of ripening demonstrated here was not accompanied by an impairment of their gustatory value or their appearance. On the other hand, it should be noted that, surprisingly, the ripening of these test cheeses stabilized: no appreciable change was observed between the 30th and the 45th day of ripening. This latter result is remarkable; it indicates that such cheeses

could be delivered after only 30 days in the cheese ripening cellar and could be put on sale without change for an extended period. These assays of the manufacture of cheese with the aid of the compositions of stabilized protoplasts of the invention thus confirms the value of the latter for the cheese manufacturing industry.

EXAMPLE 2 :

Preparation of protoplast-based lyophilized compositions and check of the extent of lysis of the latter

1) Strains :

6 strains were used

Micrococcus sp.	CNRZ 468 (mesophile)
Streptococcus lactis	CNRZ 304 (mesophile)
Streptococcus cremoris	CNRZ 106 (mesophile)
Streptococcus diacetylactis	CNRZ 124 (mesophile)
Streptococcus thermophilus	CNRZ 302 (thermophile)
Streptococcus thermophilus	CNRZ 385 (thermophile)

2) Media, solutions and reagents :

- culture medium : medium A of example 1
- lysozyme solution : that used in example 1
- buffer for the preparation of protoplasts : PMNS buffer

Composition :

Sucrose	171 g/l
NaCl	0.584 g/l
$MgCl_2$	1.02 g/l

0.1M phosphate buffer, pH 7, to give..... 1000 ml

of the following composition :

NaH_2PO_4, H_2O : 15.6 g/l and

Na_2HPO_4, $12H_2O$: 35.85 g/l

the PMNS buffer is filtered through a membrane of porosity 0.22 µm.

CRYOPROTECTIVE MEDIA

SKIMMED MILK :

Skimmed milk is reconstituted by the addition of 1000 ml of distilled water to 100 g of SKIM MILK powder marketed by the DIECO company. It is sterilized by thermal treatment (115°C, 20 min).

0.5M SUCROSE SOLUTION :

Sucrose (MERCK) : 171 g

Distilled water : to give 1 liter

Sterilization by filtration through a 0.22 µm membrane.

0.5M MALTOSE SOLUTION :

Maltose monohydrate (PROLABO) : 180 g

Distilled water : to give 1 liter

Sterilization by filtration through a 0.22 µm membrane.

0.5M CELLOBIOSE SOLUTION :

D (+) cellobiose (FLUKA) : 171 g

Distilled water : to give 1 liter

Sterilization by filtration through a 0.22 µm membrane.

0.5M MELIBIOSE SOLUTION :

D(+) melibiose (FLUKA) : 171 g

Distilled water : to give 1 liter

Sterilization by filtration through a 0.22 µm membrane.

0.5M LACTOSE SOLUTION :

Lactose monohydrate (PROLABO) : 180 g

Distilled water : to give 1 liter

Sterilization by filtration through a 0.22 µm membrane.

0.5M TREHALOSE SOLUTION :

D(+) trehalose (SIGMA) : 189 g

Distilled water : to give 1 liter

Sterilization by filtration through a 0.22 µm membrane.

SOLUTION OF MALTODEXTRINS :

Glucidex No. 6 (ROQUETTE) : 200 g

Distilled water : to give 1 liter

The solution is not sterilized.

3) Procedure :

a) culture of the strains

The cultures were grown at a temperature of 30°C in the case of the mesophilic strains and at a temperature of 37°C in the case of the thermophilic strains. The cultures were grown in 2 l flasks containing 500 ml of culture medium (agitation : 90-120rpm) for the first five strains and in fermenters of 20 l containing 15 l of medium A for the Streptococcus thermophilus CNRZ 385 strain.

The cultures were stopped at the end of the exponential phase of growth.

b) preparation of the protoplasts

The protoplasts were prepared in accordance with protocols similar to those used in example 1.

The efficiency of protoplast formation, which was checked as previously, was always higher than 99.9%.

c) freezing and lyophilization

The samples to be lyophilized were placed in Petri dishes 90 mm in diameter in aliquots of 10 ml per dish, then frozen at a temperature of -80°C for at least two hours.

Lyophilization was then carried out for 48 hours in a lyophilizer, with heating at a temperature of 20°C for the last two hours in order to reduce residual moisture.

The lyophilizates were stored at a temperature of +4°C in vials which were stoppered but not sealed.

d) check of the extent of lysis of the protoplasts by measurement of LDH

The extent of lysis of the protoplasts before lyophilization and at different intervals afterwards was determined by means of an intracellular enzymatic marker : the lactate dehydrogenase LDH, an enzyme present in all of the bacteria used.

The extent of lysis, T ,was calculated for suspensions of protoplasts (reconstituted by the addition of distilled water in the case of the lyophilized compositions of protoplasts) by measuring the total LDH activity of the suspension (activity produced after complete lysis of the protoplasts as a result of osmotic shock) and that of the supernatant of this suspension, according to the formula :

$$T = 100 \times \frac{\text{LDH activity of the supernatant}}{\text{Total LDH activity}}$$

The principal results obtained are presented in the tables 8 and 9 below

TABLE 8

Percentage lysis of the protoplasts derived from Streptococcus thermophilus CNRZ 385 with different cryoprotective media.

Percentage lysis / Cryoprotective medium	before lyophilization	1 day after lyophilization	15 days after lyophilization	1 month after lyophilization
(*) 0.5 M maltose solution	0.4	4	4	2
(*) 0.5 M cellobiose solution	0.4	5	6	5
(*) 0.5 M lactose solution	0.3	3	3	1
(*) 0.5 M melibiose solution	0.7	6	4	5
(*) 0.5 M trehalose solution	0.3	7	6	6
0.5 M solution of maltodextrins	1,3	4	5	4
(*) 0.5 M sucrose solution	0.6	7	5	6

(*) actual osmolality of oside : 650 milliosmoles

TABLE 9

Percentage of lysis of protoplasts derived from different strains of lactic bacteria with skimmed milk or a 0.5 M maltose solution as cryoprotective medium.

Cryoprotective medium	Percentage lysis / strain	before lyophilization	1 day after liophilization
SKIMMED MILK	Micrococcus sp CNRZ 468	16	25
	Streptococcus cremoris CNRZ 106	50	100
	Streptococcus diacetylactis CNRZ 124	<10	<10
	Streptococcus lactis CNRZ 304	100	100
	Streptococcus thermophilus CNRZ 302	6	100
0.5 M MALTOSE SOLUTION	Micrococcus sp CNRZ 468	6	27
	Streptococcus cremoris CNRZ 106	35	85
	Streptococcus diacetylactis CNRZ 124	>80	100
	Streptococcus lactis CNRZ 304	25	100
	Streptococcus thermophilus CNRZ 302	1	<20

Table 8 shows a low degree of lysis for the Streptococcus thermophilus CNRZ 385 strain with the different cryoprotectors used.

Table 9 shows that skimmed milk is a good cryoprotector for the Streptococcus diacetylactis CNRZ 124 strain and that the 0.5 maltose solution is a good cryoprotector for the Streptococcus thermophilus CNRZ 302 strain.

EXAMPLE 3 : STUDY OF THE SIMPLIFICATION OF THE PROCESS FOR THE PREPARATION OF COMPOSITIONS OF STABILIZED PROTOPLASTS.

100 ml of medium A were inoculated with about 500µl of a suspension of bacteria of the Streptococcus thermophilus CNRZ 385 strain. After incubation for 6 hours, 10 ml of this preculture were then transferred into 500 ml of medium A. The culture of the bacteria was grown at a temperature of 37°C for 6 hours with gentle agitation. At the end of this period the optical density was close to 2.

The culture was then centrifuged and the cake was taken up in 500 ml of PMNS buffer (cf. example 2). The suspension obtained was centrifuged again and the cake obtained was taken up in 50 ml of PMNS buffer. A bacterial suspension was thus obtained which was then transferred to a thermostated bath at a temperature of 44°C, equipped with a magnetic stirrer. After temperature equilibration had been attained, a solution of lysozyme (that used in examples 1 and 2) was added to the bacterial suspension at a concentration of 1 g/l. The formation of protoplasts was allowed to proceed for 1 hour and 15 minutes. The efficiency of protoplast formation, verified as previously described, was about 99.5%.

Samples of the medium containing the protoplasts thus prepared were transferred to glass flasks for freezing for 2 hours at a temperature of -80°C. Some of them were subsequently lyophilized for 48 hours, with heating at 20°C during the last 2 hours in order to reduce residual moisture. The lyophilizates were stored at a temperature of +4°C in glass flasks.

The extent of lysis of the protoplasts at different stages of the preparation of the compositions of stabilized protoplasts and during their storage was determined as in example 2 by measurement of LDH. The principal results are presented in table 10 below.

TABLE 10

Percentage lysis of the protoplasts derived from Streptococcus thermophilus CNRZ 365 after freezing and/or lyophilization of the medium obtained on completion of protoplast formation.

	PERCENTAGE LYSIS
on completion of protoplast formation	0.45
when freezing is complete	1.6
15 days after freezing	2.6
30 days after freezing	1.7
on completion of lyophilization	12.3
15 days after lyophilization	9.2
30 days after lyophilization	13.5

Table 10 shows a low degree of lysis for the protoplasts of the strain CNRZ 385. Thus, the medium surrounding the protoplasts when their formation has gone to completion, which consists of a 0.5M aqueous solution of sucrose, appears to be a good cryoprotector for this strain. The other components of this medium do not seriously impair the cryoprotective effect of the 0.5M sucrose solution observed earlier (cf. example 2).

The composition of stabilized protoplasts thus obtained after lyophilization does have the disadvantage however of being hydroscopic and difficult to prepare in a powdered form. This defect can be abolished by the addition of hydrated silica, Tixosil 38 A (Rhône Poulenc) of particle size 70 µm, to the suspension obtained at the completion of protoplast formation.

It is thus possible to simplify considerably the process for the preparation of the compositions of stabilized protoplasts by the use as cryoprotector of the medium surrounding the protoplasts when their formation has gone to completion.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. Composition of stabilized protoplasts, characterized in that it consists of a suspension of bacterial protoplasts, in the frozen or lyophilized state, in a cryoprotective medium.

2. Composition according to claim 1, characterized in that the cryoprotective medium is an aqueous solution containing a sugar.

3. Composition according to claim 2, characterized in that the sugar is an oside.

4. Composition according to claim 2, characterized in that the sugar is a polysaccharide.

5. Composition according to claim 1, characterized in that the cryoprotective medium is skimmed milk.

6. Composition according to any one of claims 1 to 5, characterized in that the protoplasts are derived from a bacterial strain used for fermentation purposes in the agri-foodstuffs industries.

7. Composition according to claim 6, characterized in that the bacterial strain is a strain of lactic bacteria.

8. Process for the preparation of a composition according to any one of claims 1 to 7, characterized in that it consists in the following steps :

a) protoplast formation from bacteria

b) freezing of the suspension of protoplasts obtained in step a) in a cryoprotective medium.

c) optionally, lyophilization of the frozen suspension thus obtained.

9. Process according to claim 8, characterized in that step a) is carried out in an osmoprotective medium.

10. Process according to one of the claims 8 and 9, characterized in that the cryoprotective medium of step b) comprises the osmoprotective medium of step a).

11. Process for the conservation of bacterial protoplasts, characterized in that it comprises the following steps :

a) introduction of the protoplasts into a cryoprotective medium,

b) freezing of the resulting suspension,

c) storage of the frozen suspension until it is used.

12. Process according to claim 11, characterized in that at the end of step b) the frozen suspension is lyophilized.

Dated this 22nd day of December 1988

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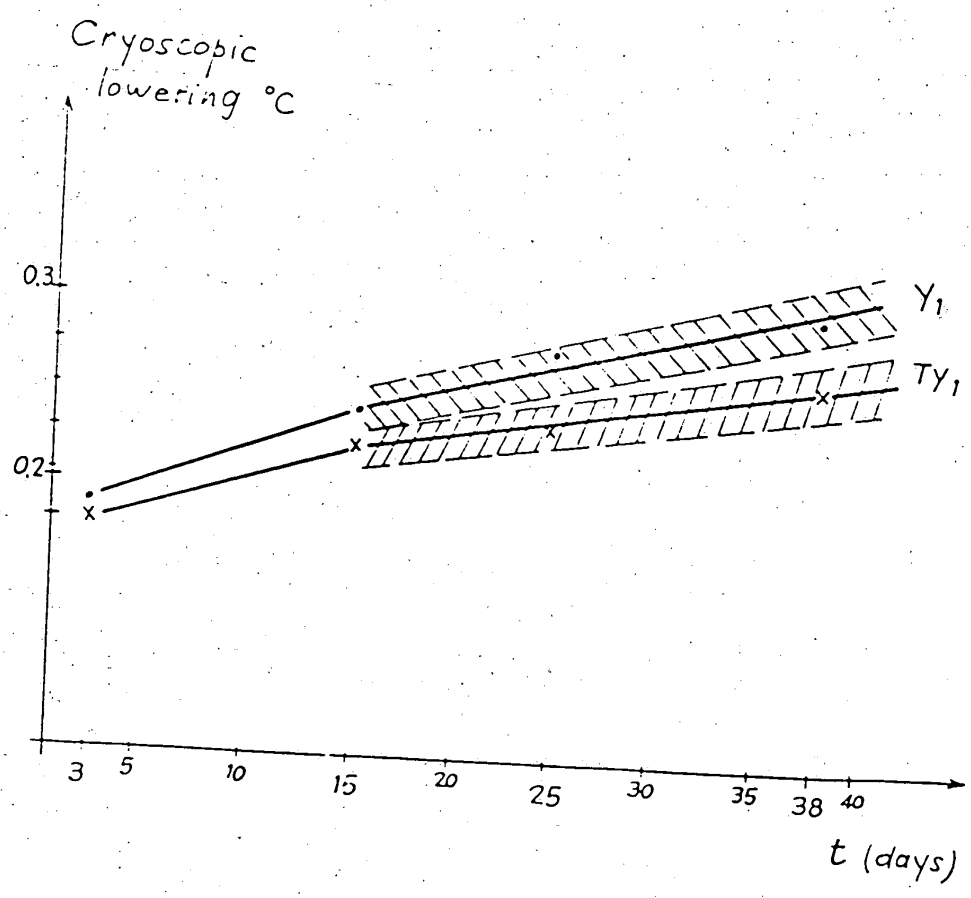
Cryoscopic lowering °C
0.3
0.2
Y_1
TY_1
3
5
10
15
20
25
30
35
38
40
t (days)